

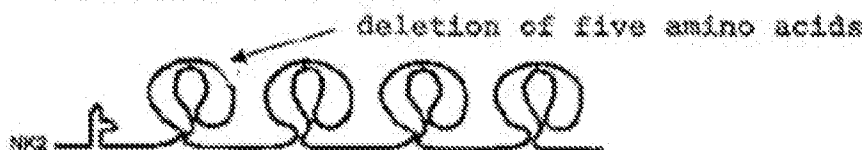
In addition, the following schematic structures of NK4 and NK4 (del 5) are shown below for clarification. It is noted that these structures contain one hairpin domain and four Kringle domains.

NK4



Total amino acids: 447

NK4(del 5)



Total amino acids: 442

Furthermore, Applicants note that the deletion of amino acids corresponding to 131-135 of SEQ ID NO:1 in SEQ ID NO:2 do not ablate the claimed inhibition of neovascularization.

According to the information from the NCBI database which is submitted herewith, positions 131 to 135 of NK4 (which correspond to positions 162-166 in HGF) are contained in the 1<sup>st</sup> Kringle domain. Positions 128-206 of NK4 correspond to the 1<sup>st</sup> Kringle domain on page 7, line 31 from the bottom to line 26 from the bottom of the information from the NCBI database.

Kringle domain forms specific a structure based on three disulfide bonds in the domain. The position of disulfide bond in the 1<sup>st</sup> Kringle domain is 128C-206C (see page 7, line 25 from the bottom to line 21 from the bottom), 149C-189C (see page 7, line 15 from the bottom to line 11 from the bottom) and 177C-201C (page 8, lines 14 to 18). These amino acids are conserved

even in the deleted form, and the formation of the disulfide bond is not destroyed. Thus, it is clear to one of ordinary skill in the art that the Kringle domain is conserved.

Regarding the last aspect of the rejection, Applicants respectfully disagree with the Office. However, solely to advance prosecution of the present application, claim 4 is canceled. Thus, the rejection with regard to claim 4 is rendered moot.

Accordingly, reconsideration and withdrawal of the rejection under § 112, first paragraph, is respectfully requested.

**Response to Rejection Under 35 USC § 102(b)**

Claim 1 is rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Date et al. The Office Action states that Date et al. disclose HGF variant HGF/NK4, which is identical to SEQ ID NO: 1, and that HGF/NK4 has a hairpin domain and four Kringle domains. The Office Action further asserts that HGF/NK4 was used to examine the mitogenic activity on rat hepatocytes in primary culture. At page 4 of the Office Action, the Office Action admits that Date et al. fails to disclose SEQ ID NO: 2.

In response, and solely to advance prosecution of the present application, claim 1 has been canceled. Thus, the rejection will be rendered moot.

Accordingly, withdrawal of the rejection under § 102(b) is respectfully requested.

**Response to Rejection of Under 35 U.S.C. § 103(a)**

1. Claim 6 is rejected under 35 U.S.C. § 103(a) as being obvious over Date et al. in view of Nakamura et al. Claims 1-2 and 4 will not be subject to the rejection if the Applicant is persuasive in making the argument set forth above (i.e., that an intact Kringle domain is present in SEQ ID NO: 2). The Office Action asserts that Date et al. disclose HGF/NK4 (SEQ ID NO: 1) in primary culture (i.e., a pharmaceutically acceptable formulation). The Office Action admits

that Date et al. does not disclose SEQ ID NO: 2. The Office Action asserts that Nakamura et al. disclose a variant of HGF comprising the 5 amino acid deletion present in SEQ ID NO: 2 and that the variant binds HGF receptors. *See*, pages 7-9 of the Office Action, wherein a sequence alignment is presented. The Office Action concludes that it would have been obvious to vary the pharmaceutically acceptable protein of Date et al. to obtain a 5 amino acid deletion mutant disclosed by Nakamura et al.

In response, and solely to advance prosecution of the present application, claim 6 has been canceled. Thus, the rejection will be rendered moot.

Accordingly, withdrawal of the rejection under § 103(a) is respectfully requested.

2. Claims 29, 32, and 35 are rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Schwall et al. in view of Date et al. and Nakamura et al.

In response, and without conceding to the rejection, claims 29, 32, and 35 have been canceled. Thus, the rejection will be rendered moot.

The Office Action asserts that claims 12-14, 28, 30-31, 33, 34, and 36 have not been included in the §112 rejection discussed above. However, the Office Action asserts that should “at least one hairpin domain and four Kringle domain” limitation be removed, these claims will also be subject to this § 103(a) rejection.

The Office Action asserts that Schwall et al. disclose cancer treatment using HGF antagonist antibodies and describe cancer broadly, including unregulated cell growth, and including cancers accompanied by increased levels of HGF or overexpression or activation of HGF receptor. The Office Action admits however, that Schwall et al. do not teach a method in which a derivative of HGF is used as the antagonist. However, the Office Action concludes that

the combination of the references renders obvious an HGF antagonist variant method wherein the variant has the amino acid sequence of SEQ ID NO: 2.

Initially, Applicants note that the limitation “at least one hairpin domain and four Kringle domain”, has not been removed from the claims. Nevertheless, in response to the Office’s comments, Applicants submit that the references, when combined, do not disclose Applicant’s invention because the use of an antagonist derived from a wild-type HGF wherein the derivative lacks 5 amino acids in the first Kringle domain is not taught or suggested by the references for at least the following reasons.

Schwall et al. does not teach or motivate one of ordinary skill in the art to combine the elements of the Applicant’s invention in the way Applicant have done because the reference explicitly states that “the function of the kringles [of HGF] has not been determined,” that “despite its serine protease-like domain, huHGF appears to have no proteolytic activity, and the precise role of the  $\beta$ -chains remains unknown,” and “it is believed that the agonist or antagonist action of HGF/NK1 may be dependent upon cell type” (see, column 2 lines 2-3, 7-11 and 49-52, respectively). Thus, based on the teaching of Schwall et al., one of ordinary skill in the art would not know or reasonably predict that a wild-type HGF derivative could be used to inhibit cancer. In fact, Schwall et al. teaches the opposite of that which the Office Action concludes, that HGF functions to stimulate growth of various tissues and cell types. Column 1, lines 19-21, Schwall et al.

Schwall et al. describes HGF receptor antagonists that are capable of specifically binding to a HGF receptor (please see column 5, lines 22-23) , and “a method of treating cancer in mammal comprising administering an effective amount of HGF receptor antagonist which is a

monovalent antibody" (see claim 1 of Schwall). However, Schwall does not teach or suggest the presently claimed polypeptide of SEQ ID NO:2, nor the inhibition of neovascularization.

Date et al. and Nakamura et al. fail to remedy the deficiency of Schwall et al., because Nakamura et al. discloses a variant of HGF that can bind and stimulate HGF receptors. In fact, Nakamura et al. state, "the present invention enables a large quantity supply of novel *physiologically active* peptide which offers the growth of hepatocytes in vitro" [emphasis added] (column 9, lines 30-36, Nakamura et al.). Thus, like Date et al. and Schwall et al., one of ordinary skill in the art would not possibly expect that a functional derivative of HGF would inhibit anything.

Moreover, Date et al. and Nakamura et al. do not describe SEQ ID NO : 2 [i.e. HGF/NK4(del 5)] and is silent about the use of SEQ ID NO:2 for inhibition of neovascularization, as well as for treatment of a disease associated with abnormal angiopoiesis, treatment of a solid cancer and/or cancer metastasis, inhibition of tumor growth or metastasis, treatment of a disease arising from vascular hyperplasia and/or caused by an excessive or abnormal stimulation of the endothelial cells and controlling of conception.

Date et al. merely discloses that HGF is likely to be one factor which stimulates neovascularization. No evidence is provided that this is in fact the case. No connection is made that the HGF/NK4 disclosed in Date et al. inhibits neovascularization, even more so not only by the action of HGF but also by VEGF and bFGF different from HGF. Date et al. only describes that HGF/NK4 is an antagonist of HGE, and abrogates the mitogenic, motogenic, and morphogenic activities of HGF which does not mean that it is a potential neovascularization inhibitor. Also, as acknowledged by the Office on page 5, line 2 of the Office Action, Date et al. does not describe SEQ ID NO:2 [i.e. HGF/NK4(del5)].

Further, it would not have been obvious for a person skilled in the art to try using HGF/NK4(del 5) with a reasonable expectation of success. Date et al. does not even provide sufficient evidence that HGF in fact simulates neovascularization.

Furthermore, the polypeptide of SEQ ID No. 2 is unobvious. The polypeptide SEQ ID NO:2 [i.e. HGF/NK4(del 5)] is a peptide of 442 amino acids, and corresponds to the polypeptide SEQ ID NO:1 in which five amino acids are deficient on the first Kringle domain at positions 95-176 among four Kringle domains. Moreover, the amino acid sequence of -Phe-Leu-Pro-Ser-Ser- is deficient at positions 131 to 135 on the first Kringle structure. Such structure of polypeptide of SEQ ID NO:2 is not obvious over the polypeptide HGF/NK4 described in Date et al.

A person skilled in the art would not have expected that such a specific modified peptide would be functional as a neovascularization inhibitor.

Nakamura et al. describes HGF having the amino acid sequence of the 1st Met to the 728th Ser in Fig. 2 and HGF having the amino acid sequence of the 1st Met to the 723rd Ser in Fig. 3 (see e.g. claims 2 and 3). However, Nakamura et al. neither describes nor suggests the inhibitory activity of neovascularization as well as the polypeptide of SEQ ID NO:2.

For at least the reasons discussed above, the inhibition of neovascularization by the polypeptides of SEQ ID NO:2 of the present invention is not obvious.

Accordingly, reconsideration and withdrawal of the rejection under § 103(a) is respectfully requested.

**Conclusion**

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,

/Tu A. Phan/

SUGHRUE MION, PLLC  
Telephone: (202) 293-7060  
Facsimile: (202) 293-7860

WASHINGTON OFFICE

**23373**

CUSTOMER NUMBER

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Tu A. Phan-Kerr, Ph.D.  
Registration No. 59,392

Date: February 14, 2008

# Kringle Structures and Antiangiogenesis

Yihai Cao\*, Renhai Cao and Niina Veitonmäki

*Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm, Sweden*

**Abstract:** The quiescent vascular system in the adult body represents the balanced net outcome of overproduction of endogenous angiogenesis inhibitors and reduced levels of angiogenic factors. While these inhibitors are expressed under physiological conditions, they are also generated in association with tumor growth. Angiostatin is such a specific angiogenesis inhibitor produced by tumors. It inhibits primary and metastatic tumor growth by blocking tumor angiogenesis. Encouraged by its potent anti-tumor activity, angiostatin is in clinical trials for cancer therapy. Angiostatin contains the first four triple loop structures, known as kringle domains, of plasminogen. The disulfide bond-linked kringle architectures are essential for the antiangiogenic activity of angiostatin. Based on this initial finding, recent work shows that kringle fragments of several other proteins also inhibit angiogenesis. Thus, kringle domains may provide a structural basis for identification of novel angiogenesis inhibitors. Surprisingly, most kringles only inhibit angiogenesis when cleaved as fragments from their parental proteins that lack antiangiogenic activity. These findings suggest that they are cryptic fragments hidden in large protein molecules. Thus, proteolytic processing plays a critical role in down regulation of angiogenesis. The kringle structure may provide the first example of a conserved architecture that specifically inhibits blood vessel growth. This review will focus on the structural and functional relationships of kringle domains in regulation of angiogenesis and tumor growth.

## TUMOR ANGIOGENESIS

Tumor growth and metastasis have been proved by many elegant experiments to be dependent on neovascularization [1-4]. The most simple and convincing piece of evidence is an experiment where a tiny piece of tumor tissue was implanted in the rabbit cornea [1]. The tumor implant changed its growth rate from linear to exponential when newly formed blood vessels reached the tumor tissue [1]. At the prevascular state, a tumor tissue consisting of several millions of cells remains in its dormant stage and the tumor implant is unable to grow beyond volumes of 2-3 mm<sup>3</sup> for about 10 days. Survival of tumors cells in an avascular tumor is dependent on free diffusion of nutrients, O<sub>2</sub> and growth factors. The living tumor cells can still produce potent angiogenic factors, such as vascular endothelial growth factor/vascular permeability factor (VEGF/VPF) and fibroblast growth factor-2 (FGF-2), which switch on an angiogenic phenotype of the tumor implant. Once newly formed blood vessels reach the tumor implant, the growth of the tumor becomes exponential. In fact, the tiny tumor implant grows beyond the size of the entire eye organ within a couple of days. However, mechanical disruptions of tumor-induced vessels in the cornea can completely arrest the tumor growth.

The most striking event in this tumor model is the switch from an avascular tumor to a highly vascularized tumor. Under physiological conditions, the corneal organ itself is avascular and is thought to contain high levels of angiogenesis inhibitors. For example, pigment epithelial

cell-derived factor (PEDF) has been reported to contribute to the avascular feature of the cornea [5]. More recently, a naturally occurring dominant negative HIF-1 $\alpha$  has been found to be highly and selectively expressed in corneal epithelial cells and to inhibit VEGF-induced angiogenesis [6]. If these angiogenesis inhibitors are expressed at high levels in the cornea, how could a tiny piece of tumor tissue shift the balance from an avascular phenotype into a vascular phenotype? The exact answer to this question is unknown. However, It seems that the tiny tumor implant might not produce enough angiogenic factors to counteract high levels of inhibitors. It is possible that tumor cells have to first down-regulate levels of angiogenesis inhibitors produced by the cells themselves and by host tissues before their angiogenic factors can initiate tumor growth. This hypothesis should be further validated. In summary, this simple experiment provides some important information in addressing the role of angiogenesis in tumor growth. 1) It is evident that a tumor tissue is able to recruit new blood vessels. 2) Tumor growth is dependent on these newly formed vessels. 3) Therapeutic suppression of tumor vessels can inhibit or stop tumor growth. 4) Switch to a tumor angiogenic phenotype involves complex regulations of both angiogenic factors and inhibitors.

## ANGIOGENESIS INHIBITORS AND CLINICAL TRIALS FOR CANCER THERAPY

Less than 10 years ago, only a few compounds, including some small chemical molecules and a couple of endogenous protein molecules, were described as angiogenesis inhibitors [7]. Today, there are so many angiogenesis inhibitors being identified and reported that it is impossible to include all angiogenesis inhibitors as a one-page table. These angio-

\*Address correspondence to this author at the Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm, Sweden; Tel: (+46)-8-728 7596; Fax: (+46)-8-31 94 70; E-mail: yihai.cao@mtc.ki.se



genesis inhibitors include natural chemical compounds extracted from plants, compounds derived from microorganisms, synthetic chemicals, intact protein molecules, protein fragments and small peptides [7, 8]. They interfere an angiogenic process at various steps including endothelial cell adhesion, endothelial cell proliferation, migration, survival, matrix degradation, tube formation, and vascular remodeling [9]. Almost all known angiogenesis inhibitors have been tested for their therapeutic potentials in animal tumor models. As expected, most of them produce some effects against tumor growth although their efficacies vary according to reports from various laboratories. Encouraged by these preclinical data, there are about 50 angiogenesis inhibitors including angiostatin and endostatin that have entered into early phases of human cancer trails (Table 1 and [10]). High demand of rapidly testing these compounds in cancer patients comes from several directions. 1) Classical cancer therapeutic approaches including chemotherapy and radiotherapy lack therapeutic efficacy against most malignancies. On the contrary, these approaches can produce severe and/or lethal side effects and drug resistance. 2) Cancer patients desperately seek for alternative approaches. 3) Political and economic pressures of the health care system. 4) Ethical issues. 5) Commercial interests of drug development among pharmaceutical companies.

Although virtually all angiogenesis inhibitors exhibit certain degrees of antitumor activity in animal models, their therapeutic efficacy in human trials may be different. According to cancer therapy history, many drugs worked in animal models and failed in human trials. One of the simple answers to this discrepancy between animals and man is that animals and humans may utilize different mechanisms in responding to these drugs. As the molecular mechanisms of most angiogenesis inhibitors remain unclear, some of these inhibitors may not work in human cancers. For example, the antiangiogenic mechanisms of angiostatin and endostatin still remain unknown. Why do these molecules selectively inhibit proliferating endothelial cells but not other cell types including quiescent endothelial cells? What are endothelial cell receptors for these inhibitors? What are the signaling pathways that transduce angiostatic signals? What are pharmacokinetics of these inhibitors in animals and humans? What are the best delivery systems for cancer treatments? These issues are critically important in understanding the basic biological mechanisms of their actions and improvement of their therapeutic efficacies. Thus, future studies should focus on the basic biology and mechanisms of action of these inhibitors.

Several angiogenesis inhibitors being tested in human trials may produce the expected anticancer effects as those observed in mice. However, for some inhibitors in current trials, it is perhaps more important to proof the principle in human cancers rather than to develop products for treatment of a large number of patients. For example, in angiostatin and endostatin therapy, it is difficult to manufacture huge amounts of biological active recombinant proteins for treatment of a large number of patients. Thus, these trials are limited to a small group of patients. Another potential disadvantage of delivery of large amounts of proteins into patients is that these materials may contain toxic

contaminants from microorganisms. This dosage-related problem could be potentially overcome by development of slow-release and gene delivery systems. In fact, gene therapy approaches demonstrate that both angiostatin and endostatin are effective in animal tumor models although some controversial results have also been reported [11-14]. Sustained release of angiostatin and endostatin in animals can also increase their therapeutic efficacy by increasing protein stability and half-lives [12, 15, 16]. In addition, preclinical studies show that combinations of angiostatin therapy with other existing cancer therapies including radiotherapy and chemotherapy produce synergistic effects in experimental tumors [17-19]. These observations have provided important clues and guidelines for the ongoing clinical trials. Indeed, current angiostatin and endostatin trials are recommended as a component in combinations with other cancer therapies.

According to their mode of action, angiogenesis inhibitors can be classified as: 1) Direct endothelial cell inhibitors 2) Angiogenic factor antagonists. 3) Angiogenic factor receptor antagonists. 4) Protease inhibitors. 5) Anti-inflammatory compounds. 6) Matrix protein antagonists. 7) Cytokines. Direct angiogenesis inhibitors such as angiostatin and endostatin target genetically stable newly formed endothelial cells. They directly inhibit endothelial cell proliferation, migration or vessel formation [4, 20-22]. As these inhibitors act directly on endothelial cells, they are generally not subjected to drug resistance [23, 24]. Although they could induce endothelial apoptosis, arrest cell cycles, antagonize angiogenic activities of growth factors and inhibit activation of kinases, their detailed molecular mechanisms of action are poorly understood [25-32]. In contrast to direct angiogenesis inhibitors, angiogenic factor antagonists are a group of well-characterized and mechanistically defined angiogenesis inhibitors. For example, much of recent attention in the antiangiogenesis field has been focused on development of VEGF antagonists. As most tumors produce VEGF at high levels, development of VEGF antagonists is an obvious approach to block tumor angiogenesis. These VEGF antagonists include VEGF mRNA antisense, neutralizing antibodies to VEGF and its receptors, soluble VEGF receptors, and VEGFR-2-signalinng inhibitors [2, 33-38]. In animal models, these VEGF antagonists have produced remarkable effects in suppression of tumor growth. For example, VEGF neutralizing antibodies and soluble VEGF receptors completely prevented tumor growth [2, 36]. However, if a tumor produces other angiogenic factors such as FGF-2, the tumor may become resistant to anti-VEGF treatment. As cancer masses consist of heterogeneous populations of tumor cells that constantly undergo genetic mutations, they may switch their angiogenic profiles. In fact, an early tumor may only secrete one or two angiogenic factors whereas a large progressive tumor can produce many angiogenic factors [39]. For example, more than 50% newly diagnosed breast cancers produce only VEGF. In subsequent tumor progression, recurrences and metastases, production of other angiogenic factors including, FGF-2, TGF- $\beta$ , PlGF, PD-ECGF and pleiotrophin is switched on [39, 40]. Anti-VEGF reagents might stress tumor cells to select for colonies producing other angiogenic factors such as FGF-2. Thus, VEGF antagonists may not be effective in the

Table 1. Clinical Trials

| Drug                                  | Phase  | Sponsor                  |
|---------------------------------------|--------|--------------------------|
| <b>Protease Inhibitors</b>            |        |                          |
| Captopril                             | I/II   | Bristol-Myers S.         |
| CGS 27023A                            | I/II   | Novartis                 |
| Col-3 (metastat)                      | I/II   | CollaGenex & NCI         |
| BMS-275291                            | II/III | Bristol-Myers S.         |
| Neovastat ( $\text{\AA}$ -941)        | III    | Aeterna Lab.             |
| <b>Growth Factor Antagonists</b>      |        |                          |
| IMC-1C11                              | I      | ImClone                  |
| CEP-7055                              | I      | Sanofi-Synth & Cephalon  |
| PI-88                                 | II     | Progen Industries        |
| Mobist                                | II     | Amgen/Imclone            |
| Iressa (ZD1839)                       | I/II   | Astra Zeneca             |
| PTK787 (ZK22584)                      | I/II   | Novartis                 |
| SU-6668                               | I/II   | Pharmacia Corp. /Sugen   |
| Anti-VEGF Ab. (Avastin)               | II/III | NCI, Genentech           |
| Angiozyme (Ribozyme)                  | II/III | Ribozyme Pharm.          |
| Avicine                               | II/III | AVI Biopharma            |
| IM862                                 | II/III | Cytrane                  |
| SU101                                 | II/III | Pharmacia Corp, SUGEN    |
| Octreotide acetate (Sandostatin)      | III    | Novartis                 |
| Suramin (Metaret)                     | FDA    | Warner-Lambert & NCI     |
| <b>Direct Endothelial Inhibitors</b>  |        |                          |
| Angiostatin                           | I      | EntreMed                 |
| 2-ME                                  | I      | EntreMed                 |
| Combretastatin (CA4P)                 | I/II   | Oxigene                  |
| TNP-470                               | I/II   | TAP Pharm.               |
| Endostatin                            | II     | EntreMed                 |
| Penicillamine                         | II     | NCI, available           |
| Squalamine (MSI 1256 F)               | II/III | Genera & Magainin Pharm. |
| Thalidomide                           | II/III | NCI Celgene & EntreMed   |
| <b>Farnesyl Transferase Inhibitor</b> |        |                          |
| L-778,123                             | I      | NCI, Merck               |
| SCH66336                              | I      | Schering-Plough Corp.    |
| <b>Cytokines</b>                      |        |                          |
| IL-12                                 | I/II   | Genetics                 |
| Interferon- $\alpha$                  | III    | Available                |

(Table 1) contd....

| Drug                        | Phase  | Sponsor              |
|-----------------------------|--------|----------------------|
| <b>Anti-Integrin Agents</b> |        |                      |
| Vitaxin (medi-552, LM 609)  | II     | MedImmune Inc./Ixsys |
| EMD 121974                  | I/II   | Merck                |
| <b>Other</b>                |        |                      |
| CAI (Carboxyamido-triazole) | II/III | NCI                  |
| Celecoxib                   | I/II   | Pharmacia            |
| ImmTher                     | II     | Endorex              |
| Flavopiridol                | II     | Aventis & NCI        |
| Genistein (GCP)             | I/II   | AminoA               |

treatment of all cancers. Such a principle may also apply to other anti-single factor approaches including anti-FGF-2 and anti-PDGFs. Thus, single angiogenic factor antagonists may encounter problems due to the development of drug resistance, as tumor cells most likely may switch their angiogenic stimulators. However, a recent study has provided some exceptional evidence that VEGF plays an essential and non-replaceable role of tumor angiogenesis in a mouse pancreatic tumor model [41]. In contrast to antagonists for single angiogenic factors, general angiogenesis inhibitors that block common pathways of tumor angiogenesis could bypass drug resistance and thus prove therapeutically effective against all cancer types. We speculate that combinations of various antiangiogenic molecules could be beneficial in cancer therapy.

## ANGIOSTATIN AND FRAGMENTAL ANGIOGENESIS INHIBITORS

Angiostatin was discovered nearly 10 years ago as internal fragments of plasminogen in serum and urine of tumor bearing animals [4]. As angiostatin was chosen a single name, subsequent studies have referred angiostatin as a single species. In fact, angiostatin contains several fragments of plasminogen with different biological activities [42, 43]. Thus, it is inaccurate to consider angiostatin as a single and homogenous protein fragment. In several original reports, angiostatin fragments exhibit potent effects in suppression of primary and metastatic tumor growth in animals by specifically blocking tumor angiogenesis [44, 45]. One of the most intriguing early findings was that the parental plasminogen did not affect endothelial cell growth, angiogenesis or tumor growth, suggesting that angiostatin are cryptic structures hidden in plasminogen [4]. Consistent with this finding, it has been found that tumor cells synthesize neither angiostatin nor plasminogen. Instead, they produce various proteases that cleave circulating plasminogen to release angiostatin (Table 4). Because these proteases cleave human plasminogen at different sites, various fragments with inhibitory activity of endothelial cells were named as angiostatin. These early studies indicated that proteolysis plays an important role in suppression of

angiogenesis. However, it has raised a paradoxical central question of why tumor cells produce angiogenesis inhibitors, as tumor growth and metastasis require a positive switch of angiogenesis. There are no satisfactory answers to this question. One of the possible explanations is that tumor cells are reminiscent of their ancestor normal host cells that produce large amounts of angiogenesis inhibitors. If so, angiostatin would be one of the key negative regulators of angiogenesis in healthy tissues. The fact that angiostatin has not been found in healthy tissues suggests that it remains a tumor specific angiogenesis inhibitor. Thus, the mechanism of a switch of tumor angiogenesis is complex and involves regulation of both positive and negative regulators.

As compared with angiogenic factors such as VEGF and FGF, angiostatin fragments have relatively long half-lives in the body [46]. The different half-lives between angiogenic factors and angiostatin in the circulation were also used to explain why angiostatin-producing primary tumors inhibited the growth of their metastases but not primary tumors [47]. However, in animal tumor models, high dosages of angiostatin were administered in order to reach the maximal anti-tumor effect [4, 44]. Later studies have shown that a short fragment of angiostatin (kringle 1-3) produces more potent antitumor effect than a longer fragment (kringle 1-4) [43]. This finding has led to clinical trials of kringle 1-3 in human cancer therapy [10]. For clinical trials, several major obstacles that hamper angiostatin therapy should be overcome before a large number of cancer patients are tested. These include (1) High dosages; according to preclinical results, high amounts of angiostatin (20-100 mg/kg/day) must be administered in order to produce an anti-tumor effect. If these same dosages are used in humans, at least a few grams of angiostatin should be delivered to a patient each day. Ongoing clinical trials with angiostatin and endostatin show that administration of low doses may not be effective. Thus, high dosages of angiostatin and endostatin are being considered in the revised clinical protocols [48]. (2) Repeated administrations; Angiostatin has to be delivered to the body by injections. Due to the relative short half-life in the body, angiostatin has to be delivered repeatedly from once daily to several times a day. (3) Prolonged protein therapy; it is assumed that angiostatin therapy might be

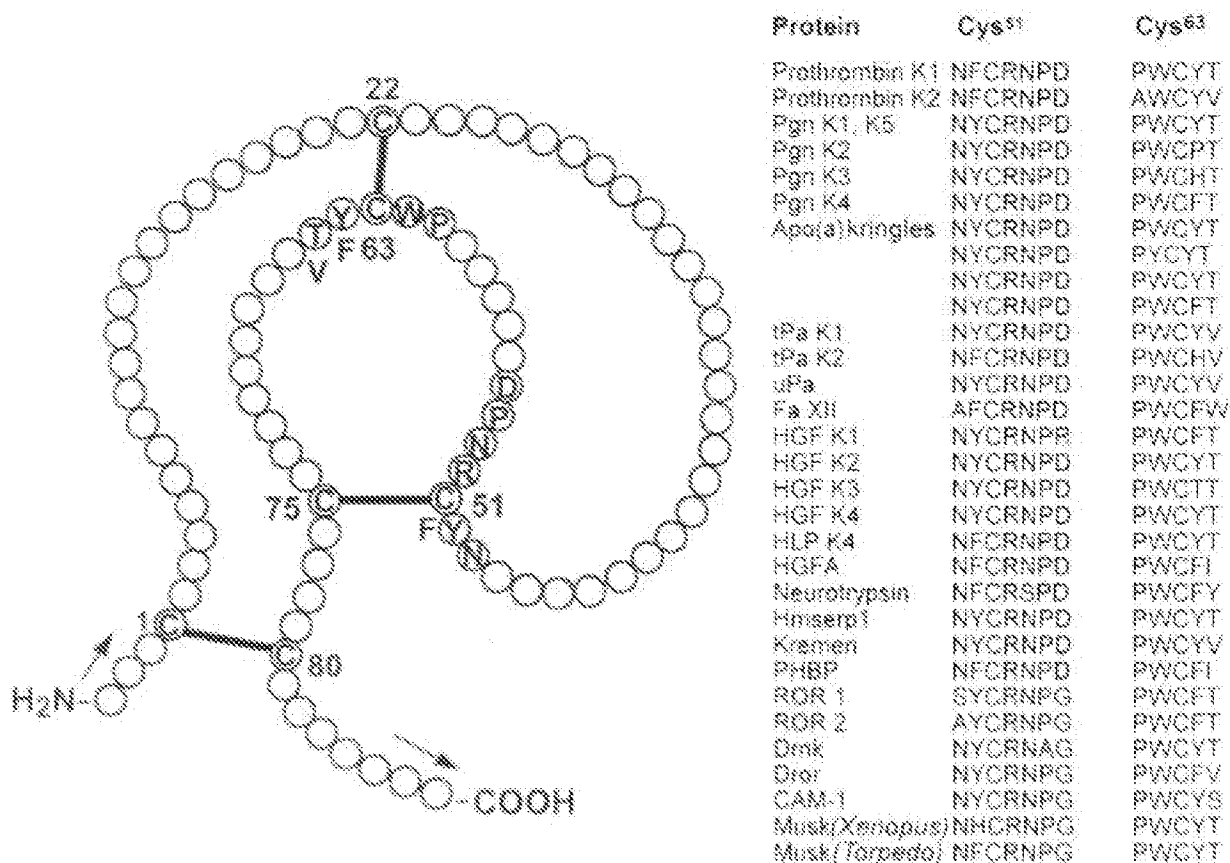
carried out for the rest of patient's life. (4) Risks of toxicity; Although angiostatin has been found to be safe and non-toxic, recombinant angiostatin produced in microorganisms could be contaminated with toxins and infectious particles, which may become harmful to the body, especially when high amounts of proteins are used. (5) High expenses; prolonged and high dose therapy requires high costs for both producers and consumers. To overcome these obstacles, alternative approaches have to be developed. These approaches may include isolation of more potent inhibitors, prolongation of half-lives, angiostatin gene therapy, targeting angiostatin into the tumor vasculature, and combination therapy with other angiogenesis inhibitors. To this end, several more potent angiostatin-related fragments including kringle 5 and kringle 1-5 have been identified [29, 42]. Sustained release of angiostatin by polymers and micropumps have shown to increase its anti-cancer efficacy [15, 16]. In addition, angiostatin gene therapy in animal models have produced promising antitumor effects in animals [11, 49].

Like many other endogenous angiogenesis inhibitors, the molecular mechanisms of angiostatic activity of angiostatin remain poorly characterized. Although great efforts have focused on identification and characterization of endothelial cell surface receptors, it is not known if some of these endothelial angiostatin-binding molecules can transduce inhibitory signals [50-53]. Likewise, the intracellular signaling molecules that mediate angiostatic activity of angiostatin also remain to be characterized. The outcome of clinical trials in human patients with angiostatin can be unpredictable due to lack of these molecular details. The antitumor activity of angiostatin observed in mice may not be necessarily identical as that seen in humans as mouse and human tumors are different. In fact, a human tumor usually takes years to become visualized whereas an experimental mouse tumor can grow into a large size within a couple of weeks [29]. Thus, it is difficult to predict its anti-tumor efficacy in humans unless the molecular mechanisms of angiostatin action are fully understood.

It appears that angiostatin is not the only cryptic fragment that inhibits angiogenesis. It has become a common theme that proteolytic fragments from large precursor proteins inhibit neovascularization. Among all known endogenous angiogenesis inhibitors, at least 50% of them are proteolytic fragments [8]. For example, many tumor angiogenesis specific inhibitors such as angiostatin, endostatin, serpin antithrombin, tumstatin, canstatin, vasostatin, prolactin, restin, and arresten are all proteolytic fragments [8, 20, 44, 54-60]. Antiangiogenic proteolysis not only indicates the complexity of regulation of angiogenesis but also implies that these angiogenesis inhibitors cannot be cloned by genetic approaches, as most of their precursor proteins do not inhibit angiogenesis. Proteolysis plays dual roles in regulation of angiogenesis. The initial process of angiogenesis requires proteolytic processing to break down the endothelial basement membrane. Whether the same protease(s) can both promote and inhibit angiogenesis is not known. However, it appears that many proteases are involved in regulation of angiogenesis.

## KRINGLE STRUCTURE

The structure of angiostatin includes the first four kringle domains of plasminogen [4]. A Kringle (*kringla* in Swedish) is a type of Scandinavian cookie folded into two rings. This term was originally adopted to describe a triple loop structure linked by three pairs of disulfide bonds present in prothrombin [61]. A typical kringle structure in a protein is shown in Fig. (1). Kringle structure exists in many proteins (Fig. 1 and Table 2) that can contain anything from one to several kringles. The primary amino acid sequence of each kringle domain is composed of about 80 amino acids. In addition to the six conserved cysteine residues in their predicted positions, amino acids flanking the third and fourth cysteines are also highly conserved (Fig. 1). However, other amino acids in the primary structure are less conserved among various kringles. Conservation of kringle structures among different proteins suggests possible replications of similar genetic information during evolution. Although kringle structures have been known for nearly 30 years, their physiological functions still remain unknown. It is clear that each kringle containing protein has its specific function. For example, they can be growth factors, proteases, or coagulation factors [61-71]. Divergence of biological functions of various kringle containing proteins might imply that these particular structures may not possess a common biological function although they may assist protein folding and stabilize other regions of proteins. Another possibility is that kringle structures can be recognized by proteases as specific cleavage sites. Although most kringle containing proteins have only one kringle domain, apolipoprotein (a) (apo [a]), a distinctive glycoprotein of lipoprotein (Lp (a), is an unusual protein in that it contains as many as 38 kringle domains (Table 2). It is not clear why this protein contains so many kringles. In addition to classical kringle structures, many proteins contain short consensus repeats (SCR unit) that constitute a similar folding module as the kringle (Table 3). The folding of the SCR unit is accomplished by the formation of two pairs of disulfide bridges, instead of three in the classical kringle domains (Fig. 2). The flanking amino acid sequences adjacent to cysteine residues do not seem to be conserved in these proteins. The biological function of the SCR unit is not known. However, this type of structural modules can mediate protein-protein interactions [72, 73]. The SCR units are often found in complement proteins and are involved in binding and thereby activation of target molecules. It is not known if SCR containing proteins or the SCR unit itself could inhibit angiogenesis. The SCR module is not the only structure that resembles the kringle architecture. Like the SCR unit, the fibronectin type II domain contains two pairs of disulfide bridges that fold this domain into a kringle-like structure (Fig. 2). Fibronectin type II domain containing proteins are found in fibronectin, matrix metalloproteinases, some receptors and seminal fluid proteins [74]. Several fibronectin type II domain-containing proteins are involved in angiogenesis. For example, fibronectin, matrix metalloproteinases and hepatocyte growth factor are involved in regulation of angiogenesis [75]. It is not clear if the fibronectin type II domain plays a role in regulation of angiogenesis by these proteins.



**Fig. (1).** Kringle architecture and conserved motifs in kringle- containing proteins. A typical kringle domain contains approximately 80 amino acids of which six cysteine residues are conserved in their predicted positions. The conserved six cysteines for a triple loop structure linked by disulfide bridges. Such a structure resembles a Scandinavian cookie named “kringla” in Swedish. In addition to the conserved cysteines, a few amino acids flanking Cys51 and Cys63 are also highly conserved among various proteins.

## ANGIOSTATIN KRINGLES

Amino acid sequence analysis of kringle domains of human angiostatin shows that K1, K2, K3 and K4 display considerable similarity (about 50% identity) [43]. Both NMR and X-ray crystallography demonstrate that the high degree of primary sequence homology translates into a remarkably uniform conformation. Among these individual kringles, K1 has been identified as the most potent inhibitor for endothelial cell growth. K3 exhibits higher inhibitory potency than K2. Surprisingly, K4 is virtually inactive in suppression of endothelial cell growth. Indeed, a short version of angiostatin that only contains the first three kringle domains without K4 (K1-3) seems to be more active than K1-4 [43] in inhibition of endothelial cell growth. It is this form of angiostatin that is in clinical trials in the treatment of human cancer. It should be cautiously emphasized that, due to its smaller size, K1-3 may have a relatively short half-life *in vivo*. Thus, *in vitro* activity should not be directly translated into the *in vivo* effects. Kringle 4 is a special segment that manifests high affinity for lysine binding [76]. In addition, K1 has been reported to bind to lysine with relatively high affinity [77]. Since K1, but not K4, is a potent endothelial inhibitor and both kringles bind to lysine, lysine binding does not seem to play a critical role in suppression of angiogenesis. Another unique feature of K4 is that it contains two clusters of positively

charged lysine residues, adjacent to cysteine 22 and cysteine 80. Inspection of the three dimensional structure reveals that these lysines, together with lysine 59 configure an exposed and positively charged area in K4, whereas other kringles lack such a cationic cluster [43, 76]. Whether this lysine-enriched domain in K4 contributes to the loss of endothelial inhibitory activity is not known. However, one could speculate that a cationic domain may interact with a negatively charged domain in another protein, such as the heparan sulfate proteoglycans (HSPGs) in the extracellular matrix. This interaction could simply sequester K4 and prevent it from endothelial inhibition.

The kringle structure is essential to maintain the anti-endothelial activity of angiostatin. Disruption of intrachain and interchain disulfide bonds by reducing reagents completely abolishes the anti-endothelial activity of angiostatin [43]. This information is important in manufacturing biologically active angiostatin and other kringle fragments for the use in antiangiogenesis. Similarly, unfolded or misfolded angiostatin or kringles expressed as cytosolic proteins should not be used in searching for angiostatin receptor(s) and studying molecular mechanisms. For example, angiostatin expressed in bacterial cells has to be appropriately refolded in order to inhibit endothelial cell growth and angiogenesis.

Table 2. Kringle Proteins

| Protein                                               | Nr of kringles | Antiangiogenesis | Reference                                                            |
|-------------------------------------------------------|----------------|------------------|----------------------------------------------------------------------|
| Prothrombin                                           | 2              | Yes              | Magnusson <i>et al.</i> , 1975 [61]                                  |
| Plasminogen                                           | 5              | Yes              | Sottrup-Jensen <i>et al.</i> , 1975 [70], 1978 [69]                  |
| uPa <sup>1</sup>                                      | 1              | N.D              | Gunzler <i>et al.</i> , 1982 [64]                                    |
| tPa <sup>2</sup>                                      | 2              | N.D              | Pennica <i>et al.</i> , 1983 [65]                                    |
| Factor XII                                            | 1              | N.D              | McMullen and Fujikawa 1985 [71] Cool <i>et al.</i> , 1985 [94]       |
| Apolipoprotein(a)                                     | 38             | Yes              | McLean <i>et al.</i> , 1987 [95] Eaton <i>et al.</i> , 1987[96]      |
| HGF <sup>3</sup> /SF <sup>4</sup>                     | 4              | Yes              | Nakamura <i>et al.</i> , 1989 [62]                                   |
| MSP <sup>5</sup> /HGF like protein                    | 4              | N.D              | Han <i>et al.</i> , 1991[63]                                         |
| HGF <sup>3</sup> activator                            | 1              | N.D              | Miyazawa <i>et al.</i> , 1993 [66]                                   |
| Kremen                                                | 1              | N.D              | Nakamura <i>et al.</i> , 2001 [97]                                   |
| Neurotrypsin/ Motopsin                                | 1              | N.D              | Gschwend <i>et al.</i> , 1997[67] Yamamura <i>et al.</i> , 1997 [98] |
| PHBP <sup>6</sup>                                     | 1              | N.D              | Choi-Miura <i>et al.</i> , 1996 [99]                                 |
| Serine protease ( <i>Hermandid momus</i> )            | 1              | N.D              | Arnold <i>et al.</i> , 1997 [68]                                     |
| ROR 1&2                                               | 1              | N.D              | Masiakowski <i>et al.</i> , 1992 [100]                               |
| <i>Drosophila</i> neurospecific receptor kinase       | 1              | N.D              | Oishi <i>et al.</i> , 1997 [101]                                     |
| <i>Drosophila</i> receptor tyrosine kinase            | 1              | N.D              | Wilson <i>et al.</i> , 1993 [102]                                    |
| <i>C.elegans</i> ROR receptor Tyrosine kinase         | 1              | N.D              | Forrester <i>et al.</i> , 1999 [103]                                 |
| Musk <sup>7</sup> ( <i>Torpedo</i> , <i>Xenopus</i> ) | 1              | N.D              | Jennings <i>et al.</i> , 1992 [104] Fu <i>et al.</i> , 1999 [105]    |

<sup>1</sup>Urokinase-type plasminogen activator, <sup>2</sup>Tissue-type plasminogen activator <sup>3</sup>Hepatocyte growth factor <sup>4</sup>Scatter factor <sup>5</sup>Macrophage-stimulating protein <sup>6</sup>Plasma hyaluronan-binding protein <sup>7</sup>Muscle specific tyrosine kinase N.D = Not Determined

## KRINGLE 5

Like other individual kringles, the K5 domain of human plasminogen contains 80 amino acid residues. Primary structure alignment shows that K5 exhibits remarkable sequence identity (about 60%) with K1 although K2, K3 and K4 also share significant homologies with K5 [42]. Similar to K1 and K4, K5 is a lysine-binding kringles of human plasminogen. Unlike K4, K5 lacks clusters of lysine residues in its primary structure. It was hypothesized that K5 might inhibit endothelial cell growth and angiogenesis. Surprisingly, both proteolytic and appropriately folded recombinant K5 displays remarkably more potent anti-endothelial effect than the other individual kringles domains. In fact, K5 alone exhibits several folds greater effect than the K1-4 of angiostatin [42]. This unexpected finding suggests that K5 might inhibit endothelial cell growth via a separate mechanism, or that K5 is a more potent inducer of inhibitory targets on endothelial cells. It is concluded from these *in vitro* studies that the ranking order of endothelial cell inhibition is K5>K1-3>K1-4>K1>K3>K2>K4. However, these *in vitro* data have not been directly translated into antiangiogenic activity *in vivo*. For example, K5 has been found to be less active than angiostatin in suppression of angiogenesis in the chick chorioallantoic membrane assay and the mouse corneal

angiogenesis model (Cao, unpublished data). Insufficient suppression of *in vivo* angiogenesis by K5 is mainly due to its relatively short half-life *in vivo*. Thus, antiangiogenic effect of a given compound must be tested in *in vivo* angiogenesis models but not only in *in vitro* endothelial cell cultures.

## K1-5

Based on the findings that both angiostatin and K5 inhibit endothelial cell growth, it was speculated that a combination of these two fragments would produce greater effect. Indeed, *in vitro* studies show that a combination of K1-4 and K5 produce a remarkably synergistic activity on suppression of capillary endothelial cell growth [29]. These results suggest that angiostatin and K5 might inhibit endothelial cell proliferation via separate pathways. This initial study stimulated the effort of obtaining a fragment of the entire kringles domain (K1-5) of human plasminogen. Urokinase-activated plasmin has been used to proteolytically release a fragment containing the K1-4 plus the most part of K5 (K1-5) of human plasminogen. Similar to a combination of angiostatin and K5, K1-5 displays approximately 50-fold greater activity than angiostatin on inhibition of endothelial cell growth. Consistent with these *in vitro* results, K1-5 has

**Table 3. Kringle Homology Proteins**

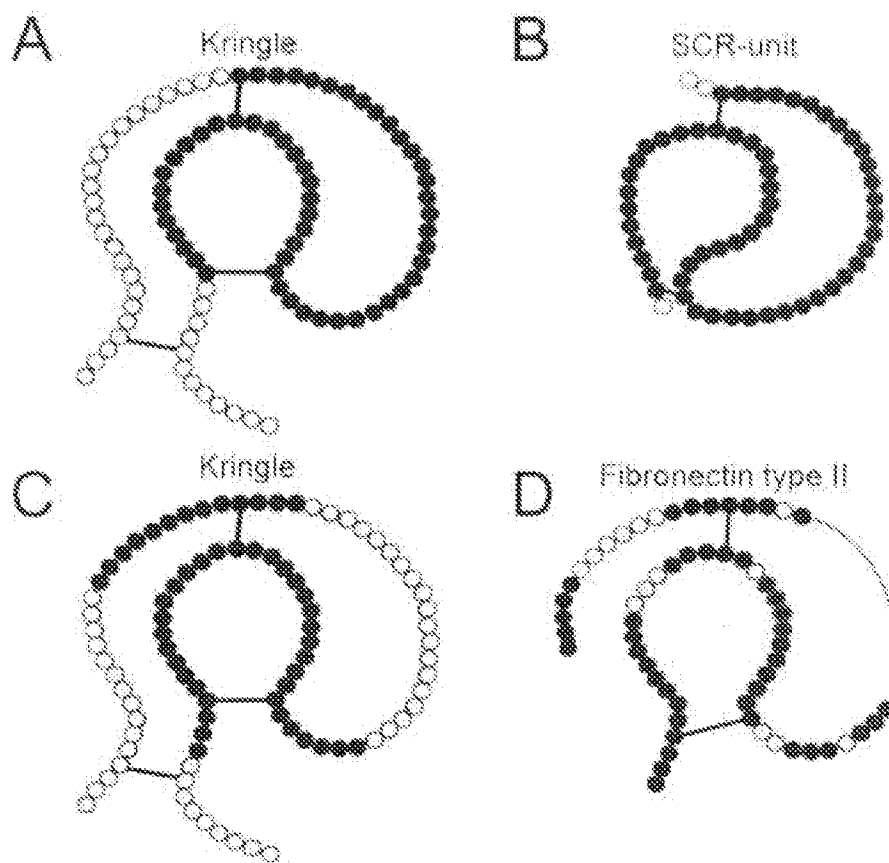
| Protein                                                              | Reference                                   |
|----------------------------------------------------------------------|---------------------------------------------|
| <b>Fibronectin type II domains</b>                                   | Patthy <i>et al.</i> , 1984 [106]           |
| Fibronectin                                                          | Petersen <i>et al.</i> , 1983 [107]         |
| PDC-109                                                              | Ecs <i>et al.</i> , 1983 [108]              |
| BSP-A3                                                               | Seidah <i>et al.</i> , 1987 [109]           |
| Mannose receptor                                                     | Taylor <i>et al.</i> , 1990 [110]           |
| Phospholipase A2                                                     | Ishizaki <i>et al.</i> , 1994 [111]         |
| DEC-205                                                              | Jiang and Nussenzweig, 1995 [112]           |
| Mannose-6-Phosphate receptor/ Insulin-like growth factor II receptor | Morgan <i>et al.</i> , 1987 [113]           |
| Pancreas-specific Sel-1                                              | Harada <i>et al.</i> , 1999 [114]           |
| proteins of vertebrates                                              | Biunno <i>et al.</i> , 2000 [115]           |
| Matrix metalloproteinase-2                                           | Collier <i>et al.</i> , 1988 [116]          |
| Matrix metalloproteinase -9                                          | Wilhelm <i>et al.</i> , 1989 [117]          |
| Factor XII                                                           | McMullen <i>et al.</i> , 1985 [71]          |
| Hepatocyte growth factor activator                                   | Miyazawa <i>et al.</i> , 1993 [66]          |
| <b>Short consensus repeats (SCR)</b>                                 | Janatova <i>et al.</i> , 1989 [118]         |
| Complement C4b binding protein                                       | Chung <i>et al.</i> , 1985 [119]            |
| Complement protein H                                                 | Kristensen and Tack, 1986 [120]             |
| Decay accelerating factor                                            | Medof <i>et al.</i> , 1987 [121]            |
| Membrane cofactor protein                                            | Lublin <i>et al.</i> , 1988 [122]           |
| Complement receptor 1                                                | Klickstein <i>et al.</i> , 1985, 1988 [123] |
| Complement receptor 2                                                | Moore <i>et al.</i> , 1987 [124]            |
| Complement component factor B                                        | Morley and Campbell 1984 [125]              |
| Complement C2                                                        | Bentley <i>et al.</i> , 1986 [126]          |
| Complement C1r                                                       | Leytus <i>et al.</i> , 1986 [127]           |
| Complement C1s                                                       | Mackinnon <i>et al.</i> , 1987 [128]        |
| Factor XIII                                                          | Lozier <i>et al.</i> , 1984 [129]           |
| Interleukin-2 receptor                                               | Leonard <i>et al.</i> , 1985 [130]          |
| Vaccinia virus secretory protein                                     | Kotwal <i>et al.</i> , 1988 [131]           |
| <b>Other homologues</b>                                              |                                             |
| Surface protein B                                                    | Johansson <i>et al.</i> , 1991 [132]        |
| Haptoglobin                                                          | Kurosky <i>et al.</i> , 1980 [133]          |

been found to produce potent antiangiogenic and antitumor activities at low doses at which angiostatin is inactive. Like angiostatin and K5, the inhibitory activity of K1-5 is restricted to the endothelial lineage but does not affect other cell types. Recently it has been found that K1-5 is a naturally occurring angiogenesis inhibitor in the body [78].

This finding suggests that K1-5 plays a role in regulation of physiological and pathological angiogenesis.

#### OTHER ANTIANGIOGENIC KRINGLE PROTEINS

In addition to plasminogen, kringle fragments derived from several other proteins have been shown to inhibit



**Fig. (2).** Kringle-like structures of the short consensus repeat and the fibronectin type II domain. Many proteins contain four conserved cysteines that form two pairs of disulfide bonds in the predicted positions (B and D). The structure of these protein domains linked by disulfide bridges are similar to those found in kringle structures (A and C).

angiogenesis *in vitro* and *in vivo* (Table 2). Among these, proteolytic kringle 1 and kringle 2 of prothrombin inhibit endothelial cell proliferation and migration *in vitro* [79, 80]. Large kringle repeats of apolipoprotein (a) inhibits *in vitro* angiogenesis assays [81, 82]. Although hepatocyte growth factor (HGF) is a potent angiogenic factor, kringle domains of HGF have been found to have the opposite activity on endothelial cells. This is an interesting example of one protein with dual functions on the vascular system. As shown in Table 2, kringle domains of most proteins have not been tested in regulation of angiogenesis. Thus, it would be interesting to study if these kringles can also inhibit angiogenesis. It should be emphasized that not all kringles inhibit angiogenesis. As mentioned earlier, K4 of human angiostatin does not inhibit or exhibits a moderate inhibitory activity on endothelial cells [43]. Thus, antiangiogenic activity of kringles is dependent not only on the appropriate folding of disulfide bonds but also on the amino acid sequence composition of individual kringles.

## ANTIANGIOGENIC PROTEOLYSIS

Although many proteins have been found to contain kringle structures (Table 2), none of these intact proteins inhibit angiogenesis. In contrast, a kringle protein such as HGF stimulates angiogenesis *in vivo* [83-85]. These results suggest that proteolysis plays a critical role in the release of angiostatic kringle fragments. Comparison of amino acid cleavage sites of various kringle fragments does not reveal a common cleavage site, suggesting that more than one protease are involved in generation of antiangiogenic kringles. Indeed, as shown in Table 4, matrix metalloproteases (MMPs), elastases and urokinase/tissue plasminogen activator (uPa/tPa)-activated plasmin are the three main classes of proteases that generate angiostatin from plasminogen, and each of them has several cleavage sites in plasminogen. Occasionally, coordination of two or more proteases is required to release an internal kringle fragment. It is not known how the enzymatic activities of these proteases are simultaneously regulated. In addition to the



**Table 4. Proteases that Release Kringles from Plasminogen**

| Enzyme                                       | Product            | Reference                                                                                                                                                                                                      |
|----------------------------------------------|--------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Matrix metalloproteinases</b>             |                    |                                                                                                                                                                                                                |
| MMP-2/ Gelatinase A                          | K1-3               | O'Reilly <i>et al.</i> , 1999 [134]                                                                                                                                                                            |
| MMP-3/ Stromelysin                           | K1-4, K5           | Lijnen <i>et al.</i> , 1998 [135]                                                                                                                                                                              |
| MMP-7/Matrilysin                             | K1-4               | Patterson and Sang 1997 [136]                                                                                                                                                                                  |
| MMP-9/ Gelatinase B/ Type IV Collagenase     | K1-4               | Patterson and Sang 1997 [136]                                                                                                                                                                                  |
| MMP-12/MME                                   | K1-3               | Dong <i>et al.</i> , 1997 [137]                                                                                                                                                                                |
| <b>Elastase</b>                              |                    |                                                                                                                                                                                                                |
| Elastase                                     | K1-4,K1-3, K4,K5   | Sottrup-Jensen <i>et al.</i> ,1978 [69] Takada <i>et al.</i> , 1988 [138],<br>Cao <i>et al.</i> , 1997 [42]                                                                                                    |
| Elastase + pepsin                            | K5, K1,K2-K3       | Novokhatny <i>et al.</i> ,1984 [139] Matsuka <i>et al.</i> , 1990                                                                                                                                              |
| Elastase + Chymotrypsin                      | K1                 | Lerch <i>et al.</i> , 1980 [140]                                                                                                                                                                               |
| Elastase + Staphylococcus Aureus V8 protease | K1                 | Motta <i>et al.</i> ,1986 [141]                                                                                                                                                                                |
| Elastase + streptokinase                     | K5                 | Vali and Pathy 1981[142]                                                                                                                                                                                       |
| <b>Plasmin</b>                               |                    |                                                                                                                                                                                                                |
| Plasmin autodigestion                        | K1,K1-3,K1-4, K1-5 | Kwon <i>et al.</i> , 2001[143], Falcone <i>et al.</i> , 1998 [144],<br>Stathakis <i>et al.</i> ,1997 [145], Kassam <i>et al.</i> , 2001[146],<br>Gately <i>et al.</i> , 1997 [22] Cao <i>et al.</i> ,1999 [29] |
| <b>Others</b>                                |                    |                                                                                                                                                                                                                |
| 13 kDa serine protease                       | K1-5               | Li <i>et al.</i> , 2000 [78]                                                                                                                                                                                   |
| 24 kDa endopeptidase                         | K1-4               | Lijnen <i>et al.</i> , 2000 [147]                                                                                                                                                                              |
| Cathepsin D                                  | K1-4               | Morikawa <i>et al.</i> , 2000 [148]                                                                                                                                                                            |
| PSA                                          | K1-4               | Heidtmann <i>et al.</i> , 1999 [149]                                                                                                                                                                           |

kringle fragments, more than a dozen proteolytic fragments have been found to inhibit angiogenesis and tumor growth [8]. These can be N-terminal, internal or C-terminal fragments of large precursor proteins. Thus, proteolytic processing is a common theme in suppression of angiogenesis. A paradoxical view is that proteases can also promote tumor angiogenesis and tumor growth. For example, MMP-2 and MMP-9 can release immobilized angiogenic factors from the matrix to interact with their receptors expressed on endothelial cells [86], while membrane type MMP-14 (MT-1 MMP) is an essential protease for physiological blood vessel development [87]. Plasmin is considered as a key protease for tumor cell invasion into neighboring tissues [88, 89]. Thus, positive and negative regulations of tumor angiogenesis by proteases are complex. Therapeutic designs of blocking the proteolytic processing of these proteases might also block generation of antiangiogenic fragments. Indeed, clinical trials with matrix metalloproteinase inhibitors have encountered severe side-effects of musculoskeletal pain and inflammation [90]. Results from Phase III trials have been disappointing and led some to conclude that MMPs inhibitors have no therapeutic benefit in human cancer, since there are still some in clinical

trials, which may not yet provide benefit. As a result, several MMP inhibitors have been withdrawn from the clinical trials. This is perhaps the first clinical lesson from “antiangiogenic therapy” without fully understanding the underlying mechanisms of antiangiogenic compounds and their roles in regulation of angiogenesis. It is almost certain that this is not the only lesson that we will learn from clinical trials with antiangiogenic compounds as there are another 50 angiogenesis inhibitors being tested in cancer patients, and most of their inhibitory mechanisms remain unknown.

## MECHANISMS

The mechanism of essentially all endogenous angiogenesis inhibitors on suppression of endothelial cell growth and tumor angiogenesis remains an enigma. Mechanistic studies of angiogenesis inhibitors have become a focus of many academics and pharmaceutical companies. Despite great efforts, little is known about endothelial cell components with which these inhibitors interact. The endothelial cell targets include endothelial cell surface

receptors, signaling pathways, activation of gene expression and signals leading to quiescent endothelial phenotypes. As a consequence, lack of molecular mechanisms has become one of the most common criticisms of reviewers to exclude publications of most research articles in high impact journals. Although several studies indicate that endothelial cell apoptosis, suppression of integrin signaling pathways, antagonistic effect of growth factor-induced signaling pathways, regulation of oncogenes and tumor suppressor genes and repression of endothelial cell cycles have been suggested to be involved in antiangiogenic activities of these inhibitors, none of the data provide compelling mechanistic evidence, especially in *in vivo* settings. However, these studies have pointed that the underlying mechanisms of endogenous angiogenesis inhibitors are complex and require many signaling players to coordinately suppress angiogenesis. As angiostatin is in clinical trials, it is essential to understand the underlying antiangiogenic mechanism. Although the answer to this question remains unknown, it is worthwhile to mention a couple of studies. Some have reported the isolation of possible receptors of angiostatin (ATPase, Angiomietin) [50, 51, 53]. Another study suggests that angiostatin could prevent the G2/M transition of endothelial cell cycle [31]. Recently, it has been demonstrated that endothelial apoptosis may play a role in mediation of antiangiogenic activity of angiostatin [25, 91, 92]. It would be interesting to see if kringle domains of other proteins can also inhibit endothelial cell growth through the same pathways.

## PERSPECTIVES

Antiangiogenesis is now considered to be a promising therapeutic approach in cancer, diabetes and chronic inflammation. Preclinical studies with various angiogenesis inhibitors have produced remarkable anti-tumor effects in animal models [23, 93]. Encouraged by these preclinical results, many angiogenesis inhibitors are in trials for human cancer therapy. Pharmaceutical companies are particularly interested in this area with strong hopes to develop effective anti-cancer drugs. As a consequence, nearly every major pharmaceutical company is running an antiangiogenic program and each company has their own antiangiogenic molecules. Thus, the search for novel angiogenesis inhibitors with therapeutic potentials is a competitive business. However, this type of business driven development may cause several potential problems. (1) The underlying antiangiogenic mechanisms of most inhibitors remain unknown. (2) A number of research laboratories and/or companies lack sufficient experience and effective assay systems in determination of angiostatic activities. (3) Long term toxicity and side-effects of antiangiogenic agents have not been fully studied. (4) Most antiangiogenic compounds are non-specific inhibitors for the newly formed blood vessels. It remains to be seen if these concerns affect cancer patients during antiangiogenic therapy. After years of experience and hard lessons learned in cancer research history, almost all cancer scientists and oncologists agree that translation from preclinical results into clinical effects is a revolutionary jump. The most common conclusive question in the past was why antiangiogenesis therapy worked in mice but not in humans. It seems that no one

could give a good answer to this question until the molecular mechanisms are fully understood. Let us hope that novel antiangiogenic compounds will not repeat this cancer research history. Angiostatin as an endothelial cell specific inhibitor seems to block a common angiogenic signaling pathway triggered by various angiogenic factors including VEGF and FGF-2. This is an important aspect in blocking tumor angiogenesis. Since tumor cells undergo frequently mutations in their genomes, they are most likely to switch their proangiogenic factors [39]. Antiangiogenic molecules that target only one particular angiogenic factor might encounter drug resistance problem. Thus inhibitors that block common angiogenic pathways should be more effective and avoid drug resistance problem. The unique structure of angiostatin has offered a possibility of searching for other structural homologues with perhaps more potent antiangiogenic activity. This type of approach has led to the identification of K5 and K1-5 of human plasminogen as more potent angiogenesis inhibitors than angiostatin [29, 42]. Such a strategy is not limited to human plasminogen, kringle domains of HGF, Apo (a) and prothrombin have been found to inhibit angiogenesis as well. There are more kringle fragments of various proteins that need to be tested for their ability to inhibit angiogenesis. Thus, the kringle structure provides the first example of discovery of novel angiogenesis inhibitors based on structural similarities.

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☐ 1: P14210. Reports Hepatocyte growth...[gi:123116]

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LOCUS P14210 728 aa linear PRI 04-DEC-2007  
DEFINITION Hepatocyte growth factor precursor (Scatter factor) (SF)  
(Hepatopoeitin-A) [Contains: Hepatocyte growth factor alpha chain;  
Hepatocyte growth factor beta chain].  
ACCESSION P14210  
VERSION P14210.2 GI:123116  
DBSOURCE swissprot: locus HGF\_HUMAN, accession [P14210](#);  
class: standard.  
extra  
accessions: A1L3U6, Q02935, Q13494, Q14519, Q8TCE2, Q9BYL9, Q9BYM0, Q9UDU6  
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sequence updated: Aug 1, 1991.  
annotation updated: Dec 4, 2007.  
xrefs: [M29145.1](#), [AAA52650.1](#), [X16323.1](#), [CAA34387.1](#), [M60718.1](#),  
[AAA52648.1](#), [D90334.2](#), [BAA14348.1](#), [X57574.1](#), [CAA40802.1](#), [M55379.1](#),  
[M73239.1](#), [AAA64239.1](#), [M73240.1](#), [AAA64297.1](#), [M77227.1](#), [AAA35980.1](#),  
[L02931.1](#), [AAAS2649.1](#), [U46010.1](#), [AAC50539.1](#), [AY246560.1](#), [AAC61091.1](#),  
[AC004960.1](#), [AAC71655.1](#), [CH236949.1](#), [EAL24189.1](#), [BC022308.1](#),  
[AAH22308.1](#), [BC063485.1](#), [AAH63485.1](#), [BC130284.1](#), [AAI30285.1](#),  
[BC130286.1](#), [AAI30287.1](#), [M75971.1](#), [AAG53459.1](#), [M75967.1](#), [M75966.1](#),  
[M75968.1](#), [M75969.1](#), [M75983.1](#), [AAG53460.1](#), [M75972.1](#), [M75973.1](#),  
[M75974.1](#), [M75975.1](#), [M75976.1](#), [M75977.1](#), [M75978.1](#), [M75979.1](#),  
[M75980.1](#), [M75981.1](#), [M75982.1](#), [JH0579](#), [1BHT](#), [1GMN](#), [1GMO](#), [1GP9](#), [1NK1](#),  
[1SHY](#), [1SI5](#), [2HGF](#)  
xrefs (non-sequence databases): RefSeq: [NP\\_000592.3](#),  
UniGene: [Hs.396530](#), IntAct: [P14210](#), MEROPS: [S01.976](#),  
GlycoSuiteDB: [P14210](#), Ensembl: [ENSG00000019991](#), GeneID: [3082](#),  
KEGG: [hsa:3082](#), H-InvDB: [HIX0033620](#), HGNC: [4893](#), HPA: [CAB010333](#),  
MIM: [142409](#), PharmGKB: [PA29269](#), Reactome: [REACT\\_604](#), LinkHub: [P14210](#),  
ArrayExpress: [P14210](#), CleanEx: [HS\\_HGF](#), GermOnline: [ENSG00000019991](#),  
GO: [0008083](#), GO: [0001837](#), GO: [0007067](#), InterPro: [IPR011359](#),  
InterPro: [IPR000001](#), InterPro: [IPR003014](#), InterPro: [IPR003609](#),  
InterPro: [IPR001254](#), InterPro: [IPR001314](#), Gene3D: [G3DSA:2.40.20.10](#),  
Pfam: [PF000051](#), Pfam: [PF000024](#), Pfam: [PF000089](#), PIRSF: [PIRSF001152](#),  
PRINTS: [PR00722](#), PRINTS: [PR00018](#), ProDom: [PD000395](#), SMART: [SM00130](#),  
SMART: [SM00473](#), SMART: [SM00020](#), PROSITE: [PS00021](#), PROSITE: [PS50070](#),  
PROSITE: [PS50948](#), PROSITE: [PS50240](#)  
KEYWORDS 3D-structure; Alternative splicing; Direct protein sequencing;  
Glycoprotein; Growth factor; Kringle; Polymorphism; Pyrrolidone  
carboxylic acid; Repeat; Serine protease homolog; Signal.  
SOURCE Homo sapiens (human)  
ORGANISM [Homo sapiens](#)  
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
Mammalia; Eutheria; Euarchontoglires; Primates; Haplorrhini;  
Catarrhini; Hominidae; Homo.  
REFERENCE 1 (residues 1 to 728)  
AUTHORS Miyazawa, K., Tsubouchi, H., Naka, D., Takahashi, K., Okigaki, M.,

Arakaki,N., Nakayama,H., Hirono,S., Sakiyama,O., Takahashi,K.,  
Gohda,E., Daikuhara,Y. and Kitamura,N.  
TITLE Molecular cloning and sequence analysis of cDNA for human  
hepatocyte growth factor  
JOURNAL Biochem. Biophys. Res. Commun. 163 (2), 967-973 (1989)  
PUBMED 2528952  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1).  
TISSUE=Placenta  
REFERENCE 2 (residues 1 to 728)  
AUTHORS Nakamura,T., Nishizawa,T., Hagiya,M., Seki,T., Shimonishi,M.,  
Sugimura,A., Tashiro,K. and Shimizu,S.  
TITLE Molecular cloning and expression of human hepatocyte growth factor  
JOURNAL Nature 342 (6248), 440-443 (1989)  
PUBMED 2531289  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1), AND PROTEIN SEQUENCE OF  
55-73 AND 495-520.  
TISSUE=Liver  
REFERENCE 3 (residues 1 to 728)  
AUTHORS Seki,T., Ihara,I., Sugimura,A., Shimonishi,M., Nishizawa,T.,  
Asami,O., Hagiya,M., Nakamura,T. and Shimizu,S.  
TITLE Isolation and expression of cDNA for different forms of hepatocyte  
growth factor from human leukocyte  
JOURNAL Biochem. Biophys. Res. Commun. 172 (1), 321-327 (1990)  
PUBMED 2145836  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1).  
TISSUE=Leukocyte  
REFERENCE 4 (residues 1 to 728)  
AUTHORS Seki,T., Hagiya,M., Shimonishi,M., Nakamura,T. and Shimizu,S.  
TITLE Organization of the human hepatocyte growth factor-encoding gene  
JOURNAL Gene 102 (2), 213-219 (1991)  
PUBMED 1831432  
REMARK NUCLEOTIDE SEQUENCE [GENOMIC DNA] (ISOFORM 1).  
REFERENCE 5 (residues 1 to 728)  
AUTHORS Miyazawa,K., Kitamura,A., Naka,D. and Kitamura,N.  
TITLE An alternatively processed mRNA generated from human hepatocyte  
growth factor gene  
JOURNAL Eur. J. Biochem. 197 (1), 15-22 (1991)  
PUBMED 1826653  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 2).  
TISSUE=Placenta  
REFERENCE 6 (residues 1 to 728)  
AUTHORS Rubin,J.S., Chan,A.M.-L., Bottaro,D.P., Burgess,W.H., Taylor,W.G.,  
Cech,A.C., Hirschfield,D.W., Wong,J., Miki,T., Finch,P.W. and  
Aaronson,S.A.  
TITLE A broad-spectrum human lung fibroblast-derived mitogen is a variant  
of hepatocyte growth factor  
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 88 (2), 415-419 (1991)  
PUBMED 1824873  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 3), AND PROTEIN SEQUENCE OF  
583-592.  
TISSUE=Lung fibroblast  
REFERENCE 7 (residues 1 to 728)  
AUTHORS Weidner,K.M., Arakaki,N., Hartmann,G., Vandekerckhove,J.,  
Weingart,S., Rieder,H., Fonatsch,C., Tsubouchi,H., Hishida,T.,  
Daikuhara,Y. and Birchmeier,W.  
TITLE Evidence for the identity of human scatter factor and human  
hepatocyte growth factor  
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 88 (16), 7001-7005 (1991)  
PUBMED 1831266  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 1).  
TISSUE=Embryonic fibroblast  
REFERENCE 8 (residues 1 to 728)  
AUTHORS Chan,A.M., Rubin,J.S., Bottaro,D.P., Hirschfield,D.W., Chedid,M.  
and Aaronson,S.A.



TITLE Identification of a competitive HGF antagonist encoded by an alternative transcript  
JOURNAL Science 254 (5036), 1382-1385 (1991)  
PUBMED 1720571  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 4).  
REFERENCE 9 (residues 1 to 728)  
AUTHORS Hartmann,G., Naldini,L., Weidner,K.M., Sachs,M., Vigna,E., Comoglio,P.M. and Birchmeier,W.

TITLE A functional domain in the heavy chain of scatter factor/hepatocyte growth factor binds the c-Met receptor and induces cell dissociation but not mitogenesis  
JOURNAL Proc. Natl. Acad. Sci. U.S.A. 89 (23), 11574-11578 (1992)  
PUBMED 1280830  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 2), AND MUTAGENESIS OF ARG-494.  
REFERENCE 10 (residues 1 to 728)  
AUTHORS Cioce,V., Csaky,K.G., Chan,A.M., Bottaro,D.P., Taylor,W.G., Jensen,R., Aaronson,S.A. and Rubin,J.S.

TITLE Hepatocyte growth factor (HGF)/NK1 is a naturally occurring HGF/scatter factor variant with partial agonist/antagonist activity  
JOURNAL J. Biol. Chem. 271 (22), 13110-13115 (1996)  
PUBMED 8662798  
REMARK NUCLEOTIDE SEQUENCE [MRNA] (ISOFORM 6).  
REFERENCE 11 (residues 1 to 728)  
AUTHORS Rieder,M.J., Livingston,R.J., Daniels,M.R., Chung,M.-W., Miyamoto,K.E., Nguyen,C.P., Nguyen,D.A., Poel,C.L., Robertson,P.D., Schackwitz,W.S., Sherwood,J.K., Witrak,L.A. and Nickerson,D.A.

TITLE Direct Submission  
JOURNAL Submitted (??-FEB-2003)  
REMARK NUCLEOTIDE SEQUENCE [GENOMIC DNA], AND VARIANTS LYS-304 AND TYR-330.  
REFERENCE 12 (residues 1 to 728)  
AUTHORS Hillier,L.W., Fulton,R.S., Fulton,L.A., Graves,T.A., Pepin,K.H., Wagner-McPherson,C., Layman,D., Maas,J., Jaeger,S., Walker,R., Wyllie,K., Sekhon,M., Becker,M.C., O'Laughlin,M.D., Schaller,M.E., Fewell,G.A., Delhaunty,K.D., Miner,T.L., Nash,W.E., Cordes,M., Du,H., Sun,H., Edwards,J., Bradshaw-Cordum,H., Ali,J., Andrews,S., Isak,A., Vanbrunt,A., Nguyen,C., Du,F., Lamar,B., Courtney,L., Kalicki,J., Ozersky,P., Bielicki,L., Scott,K., Holmes,A., Harkins,R., Harris,A., Strong,C.M., Hou,S., Tomlinson,C., Dauphin-Kohlberg,S., Kozlowski-Reilly,A., Leonard,S., Rohlfing,T., Rock,S.M., Tin-Wollam,A.M., Abbott,A., Minx,P., Maupin,R., Strommatt,C., Latreille,P., Miller,N., Johnson,D., Murray,J., Woessner,J.P., Wendl,M.C., Yang,S.P., Schultz,B.R., Wallis,J.W., Spieth,J., Bieri,T.A., Nelson,J.O., Berkowicz,N., Wohldmann,P.E., Cook,L.L., Hickenbotham,M.T., Eldred,J., Williams,D., Bedell,J.A., Mardis,E.R., Clifton,S.W., Chissoe,S.L., Marra,M.A., Raymond,C., Haugen,E., Gillett,W., Zhou,Y., James,R., Phelps,K., Iadanoto,S., Bubb,K., Simms,E., Levy,R., Clendinning,J., Kaul,R., Kent,W.J., Furey,T.S., Baertsch,R.A., Brent,M.R., Keibler,E., Flicek,P., Bork,P., Suyama,M., Bailey,J.A., Portnoy,M.E., Torrents,D., Chinwalla,A.T., Gish,W.R., Eddy,S.R., McPherson,J.D., Olson,M.V., Eichler,E.E., Green,E.D., Waterston,R.H. and Wilson,R.K.

TITLE The DNA sequence of human chromosome 7  
JOURNAL Nature 424 (6945), 157-164 (2003)  
PUBMED 12853948  
REMARK NUCLEOTIDE SEQUENCE [LARGE SCALE GENOMIC DNA].  
REFERENCE 13 (residues 1 to 728)  
AUTHORS Scherer,S.W., Cheung,J., MacDonald,J.R., Osborne,L.R., Nakabayashi,K., Herbrick,J.A., Carson,A.R., Parker-Katiraei,L., Skaug,J., Khaja,R., Zhang,J., Hudek,A.K., Li,M., Haddad,M., Duggan,G.E., Fernandez,B.A., Kanematsu,E., Gentles,S., Christopoulos,C.C., Choufani,S., Kwasnicka,D., Zheng,X.H., Lai,Z., Nusskern,D., Zhang,Q., Gu,Z., Lu,F., Zeesman,S., Nowaczyk,M.J., Teshima,I., Chitayat,D., Shuman,C., Weksberg,R., Zackai,E.H.,

Grebe,T.A., Cox,S.R., Kirkpatrick,S.J., Rahman,N., Friedman,J.M.,  
Heng,H.H., Pelicci,P.G., Lo-Coco,F., Belloni,E., Shaffer,L.G.,  
Pober,B., Morton,C.C., Gusella,J.F., Bruns,G.A., Korf,B.R.,  
Quade,B.J., Ligon,A.H., Ferguson,H., Higgins,A.W., Leach,N.T.,  
Herrick,S.R., Lemyre,E., Farra,C.G., Kim,H.G., Summers,A.M.,  
Gripp,K.W., Roberts,W., Szatmari,P., Winsor,E.J., Grzeschik,K.H.,  
Teebi,A., Minassian,B.A., Kere,J., Armengol,L., Pujana,M.A.,  
Estivill,X., Wilson,M.D., Koop,B.F., Tosi,S., Moore,G.E.,  
Boright,A.P., Zlotorynski,E., Kerem,B., Kroisel,P.M., Petek,E.,  
Oscier,D.G., Mould,S.J., Dohner,H., Dohner,K., Rommens,J.M.,  
Vincent,J.B., Venter,J.C., Li,P.W., Mural,R.J., Adams,M.D. and  
Tsui,L.C.

TITLE Human chromosome 7: DNA sequence and biology  
JOURNAL Science 300 (5620), 767-772 (2003)  
PUBMED 12690205  
REMARK NUCLEOTIDE SEQUENCE [LARGE SCALE GENOMIC DNA].  
REFERENCE 14 (residues 1 to 728)  
AUTHORS Gerhard,D.S., Wagner,L., Feingold,E.A., Shenmen,C.M., Grouse,L.H.,  
Schuler,G., Klein,S.L., Old,S., Rasooly,R., Good,P., Guyer,M.,  
Peck,A.M., Derge,J.G., Lipman,D., Collins,F.S., Jang,W., Sherry,S.,  
Feolo,M., Misquitta,L., Lee,E., Rotmistrovsky,K., Greenhut,S.F.,  
Schaefer,C.F., Buetow,K., Bonner,T.I., Haussler,D., Kent,J.,  
Kiekhäus,M., Furey,T., Brent,M., Prange,C., Schreiber,K.,  
Shapiro,N., Bhat,N.K., Hopkins,R.F., Hsie,F., Driscoll,T.,  
Soares,M.B., Casavant,T.L., Scheetz,T.E., Brownstein,M.J.,  
Usdin,T.B., Toshiyuki,S., Carninci,P., Piao,Y., Dudekula,D.B.,  
Ko,M.S., Kawakami,K., Suzuki,Y., Sugano,S., Gruber,C.E.,  
Smith,M.R., Simmons,B., Moore,T., Waterman,R., Johnson,S.L.,  
Ruan,Y., Wei,C.L., Mathavan,S., Gunaratne,P.H., Wu,J., Garcia,A.M.,  
Hulyk,S.W., Fuh,E., Yuan,Y., Sneed,A., Kowis,C., Hodgson,A.,  
Muzny,D.M., McPherson,J., Gibbs,R.A., Fahey,J., Helton,E.,  
Kettelman,M., Madan,A., Rodrigues,S., Sanchez,A., Whiting,M.,  
Madari,A., Young,A.C., Wetherby,K.D., Granite,S.J., Kwong,P.N.,  
Brinkley,C.P., Pearson,R.L., Bouffard,G.G., Blakesly,R.W.,  
Green,E.D., Dickson,M.C., Rodriguez,A.C., Grimwood,J., Schmutz,J.,  
Myers,R.M., Butterfield,Y.S., Griffith,M., Griffith,O.L.,  
Krzywinski,M.I., Liao,N., Morin,R., Palmquist,D., Petrescu,A.S.,  
Skalska,U., Smailus,D.E., Stott,J.M., Schnerch,A., Schein,J.E.,  
Jones,S.J., Holt,R.A., Baross,A., Marra,M.A., Clifton,S.,  
Makowski,K.A., Bosak,S. and Malek,J.  
CONSRTM MGC Project Team  
TITLE The status, quality, and expansion of the NIH full-length cDNA  
project: the Mammalian Gene Collection (MGC)  
JOURNAL Genome Res. 14 (10B), 2121-2127 (2004)  
PUBMED 15489334  
REMARK NUCLEOTIDE SEQUENCE [LARGE SCALE MRNA] (ISOFORMS 1; 5 AND 6).  
TISSUE=Brain  
Erratum:[Genome Res. 2006 Jun;16(6):804. Morrin, Ryan [corrected to  
Morin, Ryan]]  
REFERENCE 15 (residues 1 to 728)  
AUTHORS Miyazawa,K., Kitamura,A. and Kitamura,N.  
TITLE Structural organization and the transcription initiation site of  
the human hepatocyte growth factor gene  
JOURNAL Biochemistry 30 (38), 9170-9176 (1991)  
PUBMED 1832556  
REMARK NUCLEOTIDE SEQUENCE OF 1-208 AND 249-695 (ISOFORM 1).  
REFERENCE 16 (residues 1 to 728)  
AUTHORS Yoshiyama,Y., Arakaki,N., Naka,D., Takahashi,K., Hirono,S.,  
Kondo,J., Nakayama,H., Gohda,E., Kitamura,N., Tsubouchi,H.,  
Ishii,T., Hishida,T. and Daikuhara,Y.  
TITLE Identification of the N-terminal residue of the heavy chain of both  
native and recombinant human hepatocyte growth factor  
JOURNAL Biochem. Biophys. Res. Commun. 175 (2), 660-667 (1991)  
PUBMED 1826837

REMARK SIGNAL SEQUENCE CLEAVAGE SITE.  
REFERENCE 17 (residues 1 to 728)  
AUTHORS Shimizu,N., Hara,H., Sogabe,T., Sakai,H., Ihara,I., Inoue,H., Nakamura,T. and Shimizu,S.  
TITLE Hepatocyte growth factor is linked by O-glycosylated oligosaccharide on the alpha chain  
JOURNAL Biochem. Biophys. Res. Commun. 189 (3), 1329-1335 (1992)  
PUBMED 1462348  
REMARK GLYCOSYLATION AT THR-476.  
REFERENCE 18 (residues 1 to 728)  
AUTHORS Lokker,N.A., Mark,M.R., Luis,E.A., Bennett,G.L., Robbins,K.A., Baker,J.B. and Godowski,P.J.  
TITLE Structure-function analysis of hepatocyte growth factor: identification of variants that lack mitogenic activity yet retain high affinity receptor binding  
JOURNAL EMBO J. 11 (7), 2503-2510 (1992)  
PUBMED 1321034  
REMARK MUTAGENESIS.  
REFERENCE 19 (residues 1 to 728)  
AUTHORS Zhou,H., Mazzulla,M.J., Kaufman,J.D., Stahl,S.J., Wingfield,P.T., Rubin,J.S., Bottaro,D.P. and Byrd,R.A.  
TITLE The solution structure of the N-terminal domain of hepatocyte growth factor reveals a potential heparin-binding site  
JOURNAL Structure 6 (1), 109-116 (1998)  
PUBMED 9493272  
REMARK STRUCTURE BY NMR OF 31-127.  
REFERENCE 20 (residues 1 to 728)  
AUTHORS Ultsch,M., Lokker,N.A., Godowski,P.J. and de Vos,A.M.  
TITLE Crystal structure of the NK1 fragment of human hepatocyte growth factor at 2.0 A resolution  
JOURNAL Structure 6 (11), 1383-1393 (1998)  
PUBMED 9817840  
REMARK X-RAY CRYSTALLOGRAPHY (2.0 ANGSTROMS) OF 35-210.  
COMMENT On Mar 15, 2005 this sequence version replaced gi:87648.  
[FUNCTION] HGF is a potent mitogen for mature parenchymal hepatocyte cells, seems to be an hepatotrophic factor, and acts as growth factor for a broad spectrum of tissues and cell types. It has no detectable protease activity.  
[SUBUNIT] Dimer of an alpha chain and a beta chain linked by a disulfide bond.  
[INTERACTION] P08581:MET; NbExp=1; IntAct=EBI-1039104, EBI-1039152.  
[ALTERNATIVE PRODUCTS] Event=Alternative splicing; Named isoforms=6; Name=1; IsoId=P14210-1; Sequence=Displayed; Name=2; IsoId=P14210-2; Sequence=VSP\_009622, VSP\_009623; Name=3; IsoId=P14210-3; Sequence=VSP\_009617; Name=4; IsoId=P14210-4; Sequence=VSP\_009620, VSP\_009621; Name=5; IsoId=P14210-5; Sequence=VSP\_009617, VSP\_009622, VSP\_009623; Note=No experimental confirmation available; Name=6; Synonyms=HGF/NK1; IsoId=P14210-6; Sequence=VSP\_009618, VSP\_009619.  
[SIMILARITY] Belongs to the peptidase S1 family. Plasminogen subfamily.  
[SIMILARITY] Contains 4 kringle domains.  
[SIMILARITY] Contains 1 PAN domain.  
[SIMILARITY] Contains 1 peptidase S1 domain.  
[WEB RESOURCE] Name=Wikipedia; Note=Hepatocyte growth factor entry; URL='http://en.wikipedia.org/wiki/Hepatocyte\_growth\_factor'.  
FEATURES  
source Location/Qualifiers  
1..728  
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/db\_xref="taxon:9606"  
gene 1..728  
/gene="HGF"  
/note="synonym: HPTA"  
Protein 1..728

Region /gene="HGF"  
/product="Hepatocyte growth factor precursor"  
1..31  
/gene="HGF"  
/region\_name="Signal"  
/experiment="experimental evidence, no additional details recorded"

Region 32..494  
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/region\_name="Mature chain"  
/experiment="experimental evidence, no additional details recorded"  
/note="Hepatocyte growth factor alpha chain."  
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Region 32..33  
/gene="HGF"  
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/experiment="experimental evidence, no additional details recorded"  
/note="QR -> HK (in Ref. 2; CAA34387)."

Site 32  
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/experiment="experimental evidence, no additional details recorded"

Region 37..123  
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/region\_name="Domain"  
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/note="PAN."

Region 39..41  
/gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details recorded"

Region 42..54  
/gene="HGF"  
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/experiment="experimental evidence, no additional details recorded"

Region 60..63  
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/region\_name="Beta-strand region"  
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Region 67..76  
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Bond bond(70,96)  
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/bond\_type="disulfide"  
/experiment="experimental evidence, no additional details recorded"

Bond bond(74,84)  
/gene="HGF"  
/bond\_type="disulfide"  
/experiment="experimental evidence, no additional details recorded"

Region 77..79  
/gene="HGF"  
/region\_name="Hydrogen bonded turn"

/experiment="experimental evidence, no additional details recorded"

Region 76  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="K -> N (in Ref. 2; CAA34387)."

Region 86..90  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"

Region 91..94  
/gene="HGF"  
/region\_name="Hydrogen bonded turn"  
/experiment="experimental evidence, no additional details recorded"

Region 95..100  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"

Region 108..121  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"

Region 122..124  
/gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details recorded"

✓ Region 128..206  
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/note="Kring1 1."

✓ Bond bond(128,206)  
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Region 128..130  
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/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"

✓ Bond bond(149,189)  
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/bond\_type="disulfide"  
/experiment="experimental evidence, no additional details recorded"

Region 153  
/gene="HGF"  
/region\_name="Variant"  
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Region 156..158  
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Region 161..165  
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recorded"  
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Region 164..166  
/gene="HGF"  
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Region 180  
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/experiment="experimental evidence, no additional details  
recorded"  
/note="P -> T (in Ref. 6)."  
Region 188..192  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details  
recorded"  
Region 197..201  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details  
recorded"  
Region 206..208  
/gene="HGF"  
/region\_name="Hydrogen bonded turn"  
/experiment="experimental evidence, no additional details  
recorded"  
Region 209..210  
/gene="HGF"  
/region\_name="Splicing variant"  
/experiment="experimental evidence, no additional details  
recorded"  
/note="VE -> GK (in isoform 6). /FTId=VSP\_009618."  
Region 211..728  
/gene="HGF"  
/region\_name="Splicing variant"  
/experiment="experimental evidence, no additional details  
recorded"  
/note="Missing (in isoform 6). /FTId=VSP\_009619."  
Region 211..288  
/gene="HGF"  
/region\_name="Domain"  
/experiment="experimental evidence, no additional details  
recorded"  
/note="Kringla 2."  
Bond bond(211,288)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional  
details recorded"  
/note="By similarity."  
Bond bond(232,271)

/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional  
details recorded"  
/note="By similarity."  
Region 239..241  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details  
recorded"  
Region 247..249  
/gene="HGF"  
/region\_name="Hydrogen bonded turn"  
/experiment="experimental evidence, no additional details  
recorded"  
Region 251..253  
/gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details  
recorded"  
Bond bond(260,283)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional  
details recorded"  
/note="By similarity."  
Region 270..274  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details  
recorded"  
Region 279..282  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details  
recorded"  
Region 287..296  
/gene="HGF"  
/region\_name="Splicing variant"  
/experiment="experimental evidence, no additional details  
recorded"  
/note="TCADNTMNDT -> NMRDITWALN (in isoform 4).  
/FTId=VSP\_009620."  
Region 289..290  
/gene="HGF"  
/region\_name="Splicing variant"  
/experiment="experimental evidence, no additional details  
recorded"  
/note="AD -> ET (in isoform 2 and isoform 5).  
/FTId=VSP\_009622."  
Region 291..728  
/gene="HGF"  
/region\_name="Splicing variant"  
/experiment="experimental evidence, no additional details  
recorded"  
/note="Missing (in isoform 2 and isoform 5).  
/FTId=VSP\_009623."  
Region 293  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details  
recorded"  
/note="M -> V (in Ref. 2; CAA34387)."  
Site 294

/gene="HGF"  
/site\_type="glycosylation"  
/experiment="experimental evidence, no additional details recorded"  
/note="N-linked (GlcNAc...) (complex). /FTId=CAR\_000021."  
297..728  
Region  
/gene="HGF"  
/region\_name="Splicing variant"  
/experiment="experimental evidence, no additional details recorded"  
/note="Missing (in isoform 4). /FTId=VSP\_009621."  
300  
Region  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="L -> M (in Ref. 2; CAA34387)."  
304  
Region  
/gene="HGF"  
/region\_name="Variant"  
/experiment="experimental evidence, no additional details recorded"  
/note="E -> K (in dbSNP:rs5745687). /FTId=VAR\_019199."  
305..383  
Region  
/gene="HGF"  
/region\_name="Domain"  
/experiment="experimental evidence, no additional details recorded"  
/note="Kringle 3."  
Bond  
bond(305,383)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."  
Region  
317  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="V -> A (in Ref. 2; CAA34387)."  
Bond  
bond(326,365)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."  
Region  
330  
/gene="HGF"  
/region\_name="Variant"  
/experiment="experimental evidence, no additional details recorded"  
/note="D -> Y (in dbSNP:rs5745688). /FTId=VAR\_019200."  
Region  
333..335  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region  
336  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="E -> K (in Ref. 2; CAA34387)."



Region 341..344  
/gene="HGF"  
/region\_name="Hydrogen bonded turn"  
/experiment="experimental evidence, no additional details recorded"

Region 345..347  
/gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details recorded"

Bond bond(354,377)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."

Region 364..368  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"

Region 373..376  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"

Region 387  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="H -> N (in Ref. 2; CAA34387)."

Region 391..469  
/gene="HGF"  
/region\_name="Domain"  
/experiment="experimental evidence, no additional details recorded"  
/note="Kring 4."

Bond bond(391,469)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."

Region 396..398  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"

Site 402  
/gene="HGF"  
/site\_type="glycosylation"  
/experiment="experimental evidence, no additional details recorded"  
/note="N-linked (GlcNAc...) (complex). /FTId=CAR\_000022."

Bond bond(412,452)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."

Region 416  
/gene="HGF"  
/region\_name="Conflict"

/experiment="experimental evidence, no additional details recorded"  
/note="D -> N (in Ref. 2; CAA34387)."  
Region 420..422  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Bond bond(440,464)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."  
Region 451..458  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region 461..463  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Site 476  
/gene="HGF"  
/site\_type="glycosylation"  
/experiment="experimental evidence, no additional details recorded"  
/note="O-linked (GalNAc...). /FTId=CAR\_000023."  
Bond bond(487,604)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="Interchain (between alpha and beta chains) (By similarity)."  
Site 494  
/gene="HGF"  
/site\_type="mutagenized"  
/experiment="experimental evidence, no additional details recorded"  
/note="R->Q: Loss of activity due to absence of proteolytic cleavage."  
Region 495..728  
/gene="HGF"  
/region\_name="Mature chain"  
/experiment="experimental evidence, no additional details recorded"  
/note="Hepatocyte growth factor beta chain."  
/FTId=PRO\_0000028092."  
Region 495..721  
/gene="HGF"  
/region\_name="Domain"  
/experiment="experimental evidence, no additional details recorded"  
/note="Peptidase S1."  
Region 505  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="I -> V (in Ref. 2; CAA34387)."  
Region 508..513

Region /gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
509  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="V -> I (in Ref. 2; CAA34387)."  
Region 516..525  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Bond bond(519,535)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."  
Region 528..532  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region 533..535  
/gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details recorded"  
Region 541..543  
/gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details recorded"  
Region 544..549  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region 558  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="D -> E (in Ref. 2; CAA34387)."  
Region 559..561  
/gene="HGF"  
/region\_name="Hydrogen bonded turn"  
/experiment="experimental evidence, no additional details recorded"  
Region 561  
/gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="C -> R (in Ref. 2; CAA34387)."  
Region 563..565  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Site 566

Region /gene="HGF"  
/site\_type="glycosylation"  
/experiment="experimental evidence, no additional details recorded"  
/note="N-linked (GlcNAc...) (complex). /FTId=CAR\_000024."  
567..572

Region /gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
579..586

Region /gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
591..593

Region /gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
592

Region /gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="D -> N (in Ref. 6; AA sequence)."  
595

Region /gene="HGF"  
/region\_name="Conflict"  
/experiment="experimental evidence, no additional details recorded"  
/note="S -> N (in Ref. 2; CAA34387)."  
611..618

Bond /gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
bond(612,679)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."

Region /gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
630..637

Region /gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details recorded"  
639..645

Bond /gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."  
bond(642,658)

Region /gene="HGF"  
/region\_name="Hydrogen bonded turn"  
/experiment="experimental evidence, no additional details recorded"  
646..648

Site 653  
/gene="HGF"  
/site\_type="glycosylation"  
/experiment="experimental evidence, no additional details recorded"  
/note="N-linked (GlcNAc...) (complex). /FTId=CAR\_000025."  
Region 656..660  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region 662..664  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Bond bond(669,697)  
/gene="HGF"  
/bond\_type="disulfide"  
/inference="non-experimental evidence, no additional details recorded"  
/note="By similarity."  
Region 670..674  
/gene="HGF"  
/region\_name="Hydrogen bonded turn"  
/experiment="experimental evidence, no additional details recorded"  
Region 676..680  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region 682..690  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region 695..698  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region 704..709  
/gene="HGF"  
/region\_name="Beta-strand region"  
/experiment="experimental evidence, no additional details recorded"  
Region 710..712  
/gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details recorded"  
Region 713..720  
/gene="HGF"  
/region\_name="Helical region"  
/experiment="experimental evidence, no additional details recorded"

## ORIGIN

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661 aekigsgpce gdyggplvce qhkmrmvlgv ivpgrgcaip nrpgifvrva yyakwihkii
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